



TITLE:

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CITATION:

TAKAMATSU, Hideo ...[et al]. Histochemical Specific Demonstration of the Carboxyl Group in Tissue Sections. *Acta tuberculosea Japonica* 1956, 6(2): 69-74

ISSUE DATE:

1956-12-25

URL:

<http://hdl.handle.net/2433/51744>

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Histochemical Specific Demonstration of the Carboxyl Group in Tissue Sections

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(Received for publication September 27, 1956)

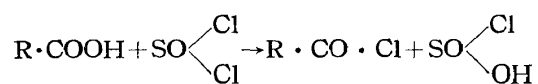
Introduction

Carboxyl group is one of the most important constituents of the animal substances. Actually, this group is an essential part of the structure of fatty acids, amino acids and other intermediary products of metabolism. There seem to be many methods devised for the histochemical demonstration of those substances in tissue section. The principles of these methods are based upon reactions other than the reaction of carboxyl group. For example, according to some books¹⁻⁴⁾, many of the histochemical or staining methods for lipids are based upon the physical or physicochemical nature of the lipids such as solubility in some media or dye solution, or upon photological phenomena. Other methods are based upon the chemical nature of unsaturated bonding of the carbon atoms in the lipid structures. Some of the other methods are based upon the reactions of the aldehyde, ketone or carbonyl group of the structures. There are also many histochemical methods for amino acids and the derivatives of proteins. Although many of these methods depend upon the reactions of some groups of those substances, there seem to be no methods depending upon the specific reaction of the carboxyl group of structures. So we intended to demonstrate the carboxyl group in tissue sections and the following method was already reported at the 45th meeting of the Japanese Pathological Society which was held on July 13, 1956⁵⁾.

Principle of the Method

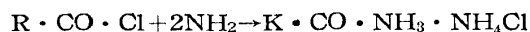
As is well known, the carboxyl group of substances can be converted to acid chloride by thionyl chloride.

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Acid chloride may form other compounds through other reactions but it is already known that thionyl chloride acts upon only the carboxyl group and does not act upon the aldehyde or the keton group. Therefore, thionyl chloride reaction is specifically for the carboxyl group. Sulphurous chloride formed in this reaction will very easily decompose into sulphurous acid and hydrogen chloride by contact with the water molecule.

Acid chloride, which formed in the first step of this method was converted into acid amide in diluted ammonia solution according to the following scheme:



This method of acid amide formation from carboxylic acid is well known as a routine technique in organic synthesis. The acid amide can be considered as a primary amine ($-\text{NH}_2$).

There are many chemical reactions of primary amine. We preferred a metaphosphate reaction for histochemical use because primary amine would form a precipitate in a concentrated metaphosphate solution, and this precipitate, i. e., metaphosphate salt was hardly soluble in water and alcohol. After the formation of metaphosphate, we tried to visualize it under the microscope.

The metaphosphate is converted into silver metaphosphate in a water solution of silver nitrate. As silver orthophosphate, the silver metaphosphate can be reduced in a neutral formol solution and will yield metallic silver. According to our new histochemical method for alkaline phosphatase and other enzymes⁶⁾, we replaced metallic silver by metallic gold in a diluted gold chloride solution. By this technique the histochemical pattern became sharper and clearer.

Procedure

1. Fixation and Sectioning: First we tried to demonstrate fatty acids of a long carbon chain. In order to do this, we fixed living tissues in an ordinary formol solution or formol solution containing 1% calcium. Then frozen sections were made. The thin sections were put on a slide glass and dried thoroughly. Unfixed and free sections or undried sections are not suitable for the following procedure because those sections are easily destroyed.

2. The slide glass with the dried section was immersed in a thionyl-chloride solution for 3~5 minutes. And leaned over a paper and dried it,

Long immersion is harmful for the sections in the following procedure. An irritative gas is emitted from thionyl chloride, so it seemed better to perform this procedure in a drafty room and to close the thionyl chloride vessel with a lid.

3. Then the slides were immersed in dilute ammonia solution (about one per cent) for 3~5 minutes. In this process, the sections will separate themselves from glass slide readily. For this reason we must do it with caution. When the gas from the ammonia solution combines with other gas from thionyl chloride in the air, a white smoke will be produced immediately. Therefore, it is recommended to keep the two vessels at a distance.

4. After the immersion in ammonia solution, the slides were dried in the air and then immersed in a concentrated liquid solution of metaphosphoric acid (freshly prepared).

5. The slide was washed in distilled water.

6. The sections were immersed in a water solution of silver nitrate (about 5 per cent) for thirty to sixty minutes.

7. The sections were washed in water again.

8. Immersing in neutralized 3 per cent formol solution (prepared by filtration of the formol solution supersaturated with CaCO_3 or $\text{MgCO}_3 + \text{Mg}(\text{OH})_2$) for 10 to 30 minutes.

9. Washed again.

10. Immersed in 0.1 to 0.05 per cent water solution of gold chloride for a few hours.

11. Counter stained with red dyes if desired. Dehydrated, cleared in xylol and mounted in canada balsam.

Results obtained

As the final product of this method, metallic gold precipitated at the locations of the carboxyl group substances and it took on a blue or blueviolet colour, according to the concentration of the matter, as seen under a microscope.

We also set up a model experiment in which an emulsion of oleic acid was injected in a mouse intraportally. Fresh slices of the liver were examined according to the method above mentioned. As is shown in the Fig. 1, many droplets of various sizes were found in this liver tissue and all of these droplets showed an intense reaction. The details of these experiments will be reported by Y. Ishiwatari, one of the present authors, in another paper. In this paper, we should like to show some of the microphotograms of the reaction of the pathologic tissues. The explanation of these plates

will be noted at the end of this report.

Comment

The principles of these histochemical reactions were mentioned above. This is based upon the fact that acid chloride can be formed specifically from the carboxyl group substances through the action of thionyl chloride. There are several well known methods to form acid chloride, but they will also convert other groups of substances and arrive at the same results. Because of this we preferred the thionyl chloride method in the first step of this histochemical test.

The most important thing seems to be what sort of substances will have this histochemical reaction. As was clearly shown in the model experiment, fatty acids of high molecular weights such as oleic acid had an intense reaction. In the experiment recorded in this report, we fixed the tissue slices in a formol solution which contained calcium ion. Fatty acids of lower molecular weights and other substances of the carboxyl group are supposed to vanish from the sections. For the determination of these substances, various fixation techniques may be combined with this histochemical reaction. It is also anticipated that the existence of some substances respond to this reaction and can remain in the sections despite of extraction by some organic solvents.

There may be other problems regarding this histochemical technique. Inasmuch as the substances vary throughout the processes of this method, it is open to question whether the substances yielded in the intermediary stages did not exist from the outset. The authors have something to relate about this interesting subject, but experimental results seemed to be insufficient. Further studies are planned. At any rate, the facts obtained by this method seemed very interesting to us.

Summary

The authors devised a method of histochemical demonstration of carboxyl groups in tissue sections. This method is dependent upon the principle of specific formation of acid chloride from the carboxyl group through the action of thionyl chloride in the first step of the method. Newly formed acid chloride was converted into acid amide in a dilute ammonia solution in the second step. The acid amide was precipitated as a metaphosphate in a concentrated sod. metaphosphate solution in the third step. Thereafter, metaphosphate was converted into silver salt in a silver nitrate solution. The silver salt was reduced to metallic silver by the neutral formol solution. The metallic silver was replaced by metallic gold by im-

mersing in a gold chloride solution to improve observation under a microscope.

There were some problems encountered in this method which are described in the item of comment of this report. Further investigations will be performed by the authors.

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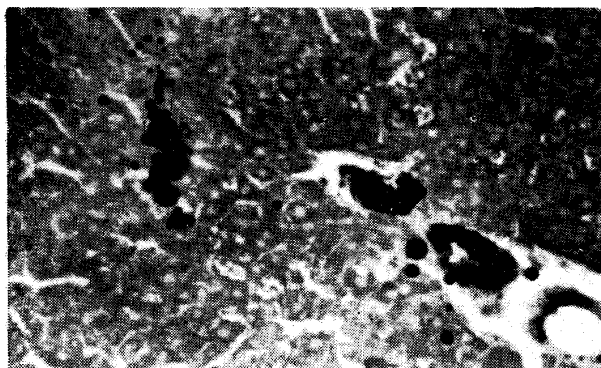


Fig. 1 : Microphotogram of the liver of the rat which was injected an emulsion of oleic acid. Stained by the method of the histochemical demonstration of carboxyl group reported in this paper. Many droplets of various sizes in the tissue were stained dark blue or blue-violet intensely.



Fig. 2 : A tuberculous lesion of a human case. This material was fixed in an ordinal formol solution and followed this new technique. Caseous area is intensely stained.

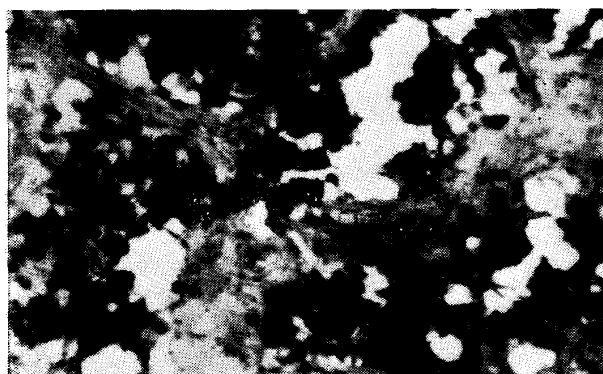


Fig. 3 : Lung tissue obtained from an autopsy case of sarcoma with metastasis in the lung. Tumor cells intensely took the reaction.



Fig. 4 : Liver tissue with metastasis of carcinoma. Carcinoma cells showed an intense reaction.